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## **Withdrawal Period of Oxolinic Acid in Cobia (*Rachycentron canadum*): Validation of an LC-MS/MS Method**

**Chen RS<sup>1</sup>, Sheu SY<sup>2,3</sup>, Xue YJ<sup>4</sup>, Wang CY<sup>5,6</sup>, Liu CH<sup>7</sup>, Kuo TF<sup>8#</sup>,  
Wang JH<sup>4#</sup>, Chou CH<sup>1#\*</sup>**

<sup>1</sup> Graduate Institute of Veterinary Medicine, National Taiwan University, Taipei 106, Taiwan

<sup>2</sup> School of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

<sup>3</sup> Department of Integrated Chinese and Western Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan

<sup>4</sup> Department of Veterinary Medicine, National Chiayi University, Chiayi 600, Taiwan

<sup>5</sup> Department of Cell Biology and Anatomy, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan

<sup>6</sup> Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan

<sup>7</sup> Department of Veterinary Medicine, National Taiwan University, Taipei 106, Taiwan

<sup>8</sup> Department of Post-Baccalaureate Veterinary Medicine, Asia University, Taichung 413, Taiwan

**Key words:** Cobia; depletion study; oxolinic acid; LC-MS/MS

### **Abstract**

Oxolinic acid (OXO) has been routinely used in aquaculture as both prophylactic and chemotherapeutic agents, principally against systemic bacterial infections. The residue depletion of OXO was investigated in cobia following multiple (5 days) in-feed dosing (30 and 60 mg/kg body weight/day) at 25°C; we assessed OXO concentrations of liver and muscle (+skin) samples post-treatment for three weeks. The OXO residues were quantified using a validated ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method and the results indicated recoveries of 86.6-95.8%, with relative standard deviations (RSD) less than 8.5% for intra- and inter-day precisions. Within the range of 10–500 ng/g, the calibration curves for all matrices presented a determination coefficient greater than 0.99. Limit of detection and quantification were estimated at 6 and 10 ng/g, respectively. In order to account for the high variability of the residue data, a safety span corresponding to 50% of depletion time could be seen as appropriate. Considering a maximum residue limit (MRL) of 50 ng/g established by the Taiwan FDA for OXO in muscle with skin in natural proportions, a withdrawal period of 15 days was calculated for the sum of 10-day depletion time and 5-day safety span. These results revealed that the analytical method is developed for residue OXO and that the withdrawal period is appropriate.

\* Corresponding author. Chung-Hsi Chou, e-mail: cchou@ntu.edu.tw

# These authors contributed equally to this work

## Introduction

Aquaculture production is one of the most relevant sources of food protein production worldwide. Cobia (*Rachycentron canadum*), have aroused high interest in the aquaculture sector and scientific community, and are an emerging species for marine aquaculture from the Indo-Pacific waters to the southern Atlantic Ocean. Due to its favorable characteristics such as excellent meat quality, rapid weight gain, and easy spawning in captivity, in both domestic and international markets, technology for its culture has rapidly developed, including mass propagation, natural spawning of captive brood stocks, larval rearing techniques, nursery production in tanks, in open ponds, near shore and offshore cages in Taiwan (Liao et al., 2004). However, aquatic animal diseases have become a major constraint inhibiting their aquaculture production. The major infectious diseases affecting cobia include bacterial (pasteurellosis, vibriosis and streptococcosis), parasitic myxosporidea, *Trichodina*, *Neobenedenia* and *Amyloodinium* infestations, and viral lymphocystis (Liao et al., 2004). It is therefore important that therapeutic regimens are designed to maximize efficacy and minimize the risk of the development of resistant pathogens.

The benefit of improved productivity of veterinary drugs in comestible animals is often associated with the risk with drug residues in the tissues of treated animals at the time of slaughter or residues due to improper drug usage and failure to keep the withdrawal period. Thereby there is a legal requirement that drug concentrations do not persist at a level greater than those established as safe by the relevant regulatory authority in the country of origin (Beyene, 2016). The highest level is referred to as the maximum residue level (MRL), that is legally tolerated in food of animal origin when veterinary drugs are applied correctly.

Many of the countries involved in aquaculture have established mechanisms for veterinary drug approval and monitoring process in aquaculture. The European Medicine Agency (EMA) regulates the licensing and authorization of drug usage in aquaculture for countries under EU. The Codex Alimentarius Commission (CAC) is a joint FAO/WHO commission charged with developing standards for food safety with worldwide applications. In Taiwan the application of drugs in aquaculture is regulated by the 'Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ)' to ensure safe and effective drugs/medicines for use in aquaculture.

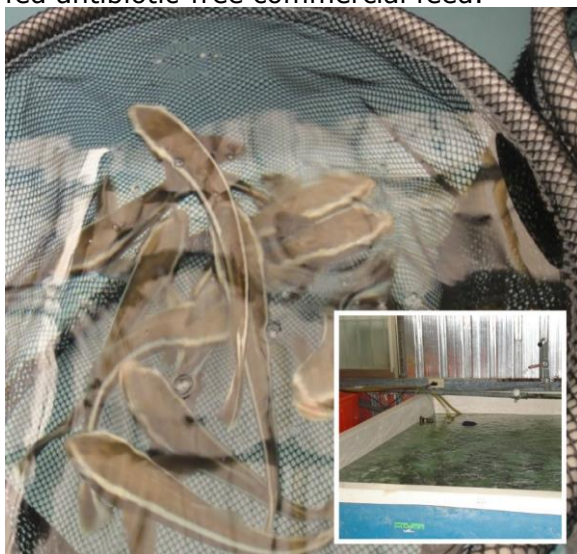
Quinolones such as oxolinic acid (OXO), have a wide bioavailability, relative low toxicity, and favorable pharmacokinetics, and have been extensively used in aquaculture against bacterial infections including pasteurellosis, vibriosis and streptococcosis, etc. (Sellyei et al., 2009; Touraki et al., 2012; Dangwetngam et al., 2016), by increasing the concentration of drug-enzyme-DNA cleavage complexes and converting them into cellular toxins to turn them into bactericidals (Naeem et al., 2016). OXO is authorized in veterinary medicine for use in fin fish. The recommended doses are 12 mg/kg bw/day for up to 7 days for the treatment of gastro-enteritis due to *Escherichia coli* as set by European Medicines Agency (EMA) (2005). Permitted limits, MRL, of OXO are 100 ng/g allowed by the European Union (EU) established for muscle and skin in natural proportions (EU, 2009).

Though a number of residue studies for OXO have been published (Samuelsen et al., 1992; Guo and Liao, 1994; Rigos et al., 2003; Gonzalez et al., 2010), no previous work has investigated Cobia (*R. canadum*). This study was undertaken to establish the withdrawal time following OXO treatments of cultured cobia to depletion below 50 ng/g in muscle and skin (the current tolerance level set by the Taiwan Food and Drug Administration (Taiwan FDA; TFDA) (TFDA, 2014). An effort was made to standardize the administration of the drug as well as the sampling and analytical protocol. The results obtained from these drug depletion trials suggest that public policies should impose regulations to ensure the proper use of OXO in cobia farms to further protect the health of consumers and maintain high export safety.

## Materials and Methods

### *Animal management.*

Healthy cobia (mean weight, 140-170 g) obtained from Fanliao aquafarms, (Pingtung, Taiwan), had been maintained at 25°C in fiber-glass-reinforced plastic (FRP) tanks of 1.8 ton (1.2 × 2.0 × 0.75 m) capacity with flow-through water systems during the experiments (Fig. 1). Fish were fed either medicated or nonmedicated commercial feed at the rate of 1% of fish body weight once each day in the morning. All animal studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee at National Taiwan University (103-EL-20) and were carried out in accordance with the provisions of the EC Council Directive 2010/63/EU. Before the onset of drug administration, the cobia were acclimated for at least 2 weeks during which they were fed antibiotic-free commercial feed.



**Fig. 1.** Fiber-glass-reinforced plastics tank used for fish farming in residue depletion study.

### *Study design.*

Medicated and unmedicated feed products (Oxalyl powder®) were obtained from Juily Pharmaceutical Co., LTD (New Taipei City, Taiwan). Animals in the control group (no OXO added) were processed simultaneously. The OXO antibiotics concentration in feed was calculated to obtain an intake equivalent to a dose of 30 or 60 mg/kg of body weight. All medicated feeds were provided for 5 days. The low dose of 30 mg/kg/day was selected based on empirical, positive impressions of clinical efficacy in aquatic species. The high dose of 60 mg/kg/day was chosen to monitor safety at the 2 dose levels within the trial. In this study, the experimental protocol included the sampling of fish at 1, 3, 5, 7, 10, 14 and 21 days after the 5-d medication period. Five fish were randomly sampled at each time point and sacrificed to analyze the residues of OXO in selected tissues (liver and muscle-skin samples). Muscle and the overlying skin were dissected from the mid-trunk region near the lateral line. The sampled area was descaled prior to excision from the fish and were kept frozen at -20°C until being analyzed.

### *Sample preparation.*

Antimicrobials were extracted from aquatic tissue using an extraction procedure based on less organic solvent consuming methodology. A 5 g homogenate fish sample (muscle-skin/liver) was mixed with 25 mL of acetonitrile containing a 5% of methanol, and homogenized with a 12-speed osterizer blender (Oster, US) for 3 min. Then 10 g of anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) was added, and the mixture was shaken using a Cute Mixer (CM-1000; Eyela, Japan) for 10 min and centrifuged (5804R, Eppendorf, Germany) at 3,500  $\times g$ , for 10 min at 4°C. The supernatant was then transferred into a clean tube. The extraction procedure was performed twice; then the two extracts were combined and transferred into a separatory funnel and 30 mL of *n*-hexane saturated with acetonitrile was added to the solution and the contents were shaken vigorously for 3 min in a

reciprocating shaker. The acetonitrile layer was then transferred to a vacuum rotary evaporator flask, and concentrated to dryness at 40°C. Finally, the residue was redissolved in methanol (50%) to make exactly 1 mL of sample solution, and this was filtered through a 0.22 µm nylon membrane filter, followed by LC-MS/MS analysis.

*Determination of OXO residues in cobia by qualitative LC-MS/MS.*

The chromatographic analyses were performed on an Acquity UPLC system, and separations were achieved using an Acquity UPLC® HSS C18 column (1.7 µm, 2.1 × 100 mm; Waters, US). The column temperature was set at 35°C. The mobile phase flow rate was fixed at 0.2 mL/min and the following program was applied for the elution:

0 min, 96% A; 0–2 min, 96–95% A; 3.0–6.0 min, 80–75% A; 6.0–8.6 min, 75–73% A; 8.6–14.5 min, 73–63% A; 14.5–14.7 min, 63–0% A; 14.7–18.7 min, 0–96% A and 18.7–20.0 min, 96% A. Deionized water (DI water):formic acid (99.9:0.1, v/v) as eluent A and methanol: formic acid (99.9:0.1, v/v) as solvent B. Injection volume was 10 µL.

Mass spectrometry analyses were carried out using a Waters Acquity TQS™ Micromass Quattro Ultima triple-quadrupole MS quadrupole equipped with an electrospray ion source (Micromass, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive mode with the following parameters: 3.3 kV capillary voltage, 25 V cone voltage, 120°C source temperature, 450°C desolvation temperature, 100 L/h cone gas flow and 800 L/h desolvation gas. The OXO isomers were fragmented using collision-induced dissociation and the multiple reaction monitoring (MRM) transitions  $m/z = 262 > 244$ ,  $m/z = 262 > 216$  and  $m/z = 262 > 160$  were monitored ( $m/z$ , mass-to-charge ratio). Data acquisition was performed using MassLynx V 4.1 software with the Quanlynx program (Waters).

*Quality control.*

The method was validated with the following performance parameters: linearity and linear range, detection and quantitation limits. The intra-day and inter-day precision of the method, expressed as the relative standard deviation (RSD) of known concentrations measurements, were assessed by using blank fish samples fortified with OXO at three concentrations of 10, 50, and 100 ng/g. Five replicates determination of each concentration was conducted during routine operation of the system over a period of five consecutive days. Internal quality controls were used, e.g., a blank extract was used to identify false positive results obtained through contamination from the extraction process, instruments, or chemicals, and to identify possible matrix interferences. Another blank sample was spiked with OXO at 10 ng/g (muscle) and 20 ng/g (liver) and the extraction efficiencies were assessed. A matrix-matched calibration curve was prepared daily from the blank matrix extract.

## Results

*Methods validation*

The developed procedure was designed to obtain qualitative and quantitative surveillance information about the quinolone antibiotic and to simultaneously analyze the same analytical protocol. The identification of OXO by LC-ESI-MS/MS obtained from fortified blank fish muscle-skin and liver samples with retention times ranging from 18.302 to 18.363 minutes. Linear regression coefficients ( $R(2)$ ) of matrix-match calibration standard curves established for quantification were higher than 0.9989, which is adequate for analytical purposes. Inter-assay precision with percentage relative standard deviations (RSD) was in the range 2.3–8.5%. Intra-day precision (RSD) was lower than 5.6%. The limit of detection (LOD) was 6 ng/g given by signal-noise ratio  $\geq 3$  (S/N) and the limit of quantification (LOQ,  $S/N \geq 10$ ) was estimated at 10 ng/g. Average recoveries and extraction efficiency were synonyms of the fortified OXO at two concentrations 10 and 20 ng/g were between 86.6% and 95.8%, with RSD in the range of 1.4–4.2%.

*Residue depletion studies*

In the 5-day repeated dose study in cobia, OXO was performed using doses of 30 and 60 mg/kg bw/day in the low- and high-dose groups. Results for OXO depletion from fish tissues (muscle plus skin in natural proportions and liver) at different times are shown in Table 1. The OXO concentrations were below the MRL (50 ng/g) established by the Taiwan FDA: range 30–40 ng/g in the muscles (at day 5), and 10–30 ng/g in the liver

(at day 5), respectively, for cobia in the low-dose groups; whereas range 10-30 ng/g in the muscles (at day 7), and 20-30 ng/g in liver (at day 10), respectively, in the high-dose groups.

**Table 1.** Oxolinic acid depletion at different times in cobia tissues.

Time day <sup>2</sup>	OXO concn (ng/g) <sup>1</sup> (low-dose group)			OXO concn (ng/g) (high-dose group)		
	Control	Muscle + skin	Liver	Control	Muscle + skin	Liver
1	ND <sup>3</sup>	62 ± 11.7	64 ± 20.6	ND	202 ± 44.5	264 ± 95.4
3	ND	44 ± 20.6	50 ± 11.0	ND	170 ± 23.7	33 ± 10.9
5	ND	34 ± 4.9	18 ± 7.5	ND	72 ± 11.7	42 ± 14.7
7	ND	16 ± 4.9	12 ± 4.0	ND	22 ± 7.5	40 ± 11.0
10	ND	ND	ND	ND	34 ± 8.0	24 ± 4.9
14	ND	ND	ND	ND	6.0 ± 4.9	20 ± 0.0
21	ND	ND	ND	ND	ND	ND

<sup>1</sup> Values are means ± SDs for 5 samples.

<sup>2</sup> Day after the last administration of medicated food.

<sup>3</sup> Not determined.

## Discussion

The results in this work show that OXO bioaccumulate at high concentrations in the muscle and liver, even though in the liver samples the concentrations fell below the MRL. In addition, the depletion time results agree with those reported by other researchers for depletion behavior of OXO in different species. In the case of a single oral dose of 40 mg/kg of OXO in orange-spotted grouper *E. coioides*, Guo and Liao (1994), found the elimination half-life ( $t_{1/2}$ ) value of 15.1 h at 24 °C, and OXO absorption was complete in about 12 h following dosing as well as the highest concentrations were measured in the kidney followed by the liver, muscle and serum; afterward, its concentrations declined below the MRL 10 days following medication.

Furthermore, another study showed that following OXO multiple medications of 25 mg/kg/day to salmon (*Salmo salar* L.) for ten days at 9°C, depletion times after treatment ceased for the residues of OXO concentrations which fell below the MRL of 50 ng/g after 12 days in the liver, followed by the muscle and plasma (Samuelsen et al. 1992). Similarly, another study found that the distribution and elimination of OXO in gilthead sea bream (*S. aurata*) and sharpsnout sea bream (*D. puntazzo*) following oral administration of 30 mg/kg/day on ten consecutive days at 19°C (Rigos et al., 2003). Elimination  $t_{1/2}$  were estimated to be 11-14 and 11-19 h in tissues of gilthead sea bream and sharpsnout sea bream, respectively. OXO concentration in sparids peaked on day 4, with highest concentrations in liver, followed by bile, skin, muscle and plasma, where its concentrations diminished 12 days after ceasing treatment.

As for differences in tissue distributions, it is known that biological factors affecting drug distribution are blood flow to tissue and capillary porosity (Sim, 2015). Drugs rapidly enter high perfused tissues with discontinuous capillaries (e.g. liver), while drug distribution into the tissues with low perfusion rates and with continuous capillaries (e.g. skin) is slow (Hamamoto and Mizuno, 2017). Higher OXO concentrations in muscle-skin samples than those in liver samples at the withdrawal period in the present study seem to be caused by slow drug transfer between muscle, skin, and plasma depending on the low blood perfusion in cobia muscle and skin.

Since marketing authorization applications for veterinary medicinal products for use in food producing species must include an indication of the withdrawal period, it is recommended to determine withdrawal period at the time point where the concentrations of residues in edible tissues fall below the respective MRL (EMA, 2018) for muscle and liver samples (50 ng/g). Also, the estimation of a safety span should be calculated from a half of tissue depletion time. Therefore, in order to account for the high variability of the residue data, this safety span corresponds to 50% of the 10-day depletion time; the conclusive approach would then result in a withdrawal period of 15 days as in the present study.

Our results agree with the general trend described by the aforementioned works, as on day 7 after treatment we found residue concentrations of 22 ± 7.5 ng/g in cobia

muscle-skin samples, whereas in livers (at higher doses) these were of  $24 \pm 4.9$  ng/g. Concentrations declined and fell below the MRL on day 10 following the last administration. It can be assumed that warmwater fish are likely to process drugs faster than coldwater species. Higher ambient temperatures and/or salinity may increase xenobiotic metabolism and elimination rates (Gonzalez et al., 2010), for example, OXO has been shown to be eliminated much faster in fish in seawater than those in fresh water (Ishida, 1992).

In conclusion, the analytical method for residual OXO in cobia liver and muscle was presented in our study. Our results determined by analytical methods prove that the 10-day depletion period in cobia administered medicated feed at the maximum dose and administration period of OXO (60 mg/kg/day for 5-day) is appropriate to ensure that OXO concentrations are below the MRL (50 ng/g). The depletion behavior we have detailed in this work provides data supporting more prudent use of OXO in cobia and suggests a possible withdrawal period of 15 days (10-day depletion time + 5-day safety span) after treatment. It therefore aids in the design and application of new policies of antimicrobial surveillance.

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